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Histone H4 acetylation distinguishes coding regions of the human genome from heterochromatin in a differentiation-dependent but transcription-independent manner

Laura P.O'Neill and Bryan M.Turner[1]

Anatomy Department, University of Birmingham Medical School, Edgbaston, Birmingham B15 2TT, UK

[1]Corresponding author

By immunoprecipitation of chromatin fragments from cultured human HL-60 cells with antibodies specific for H4 acetylated at specific lysine residues we have defined the level of H4 acetylation within transcriptionally active and inactive regions of the genome. H4 within or adjacent to coding regions had a similar level of overall acetylation to input (bulk) chromatin and a similar pattern of acetylation of individual lysines (i.e. 16 > 8, 12 > 5). The acetylation of H4 in coding (and adjacent) regions was not correlated with transcriptional activity and did not vary with position along the constitutively active c-*myc* gene. Turnover of H4 acetates was not selectively increased in transcriptionally active chromatin. H4 associated with centric heterochromatin or with the CCCTAA repeat of telomeric heterochromatin was infrequently acetylated (<1%) at all lysines. We conclude that nucleosomes containing acetylated H4 are scattered infrequently and possibly randomly through coding and adjacent regions and are essentially absent from heterochromatin. Induction of differentiation of HL-60 cells by exposure to dimethylsulfoxide or 12-*o*-tetradecanoylphorbol 13-acetate (TPA) did not alter the level of H4 acetylation within either the c-*myc* or c-*fos* genes or other coding regions, but did induce a transient increase in H4 acetylation within centric heterochromatin.

Keywords: acetylation/chromosome/gene expression/heterochromatin/histone

Introduction

In eukaryotic cells the histones that make up the nucleosome core particle (two each of H2A, H2B, H3 and H4) can all be modified *in vivo* by enzyme catalysed acetylation and deacetylation of specific lysine residues located in their N-terminal tails. This process is frequent, energy consuming and, like the histones themselves, has been highly conserved through evolution. The functional significance of this post-translational modification remains unclear, but circumstantial evidence has implicated it in several important events in eukaryotic cells, including chromatin assembly after replication, DNA repair and, most frequently, transcription (reviewed by Turner, 1991; Loidl, 1994; Turner and O'Neill, 1995). The continuing uncertainty over the role(s) of histone acetylation is attributable, at least in part, to the difficulty of determining the functional significance of acetylation of different histones or of different lysines on the same histone. Recent results suggest that both of these factors may be important.

A genetic approach in yeast has highlighted functional differences between the N-termini of the four core histones. Mutants carrying deletions of the N-terminal domains of H3 or H4 are both viable (double mutants are not), but show quite different phenotypes. H3 mutants allow hyperactivation of several genes dependent on the transcription factor GAL4 (Mann and Grunstein, 1992), while H4 mutants show both a loss of silencing at the mating type loci HMRa and HMLα and reduced induction of the genes GAL1 and PHO5 (Kayne *et al.*, 1988; Durrin *et al.*, 1991). Deletions of the N-terminal domains of H2A or H2B are both viable (once again the double mutant is not), but show no discernable phenotype (Kayne *et al.*, 1988). In the case of H3 and H4 the phenotypes of the deletion mutants can be reproduced by substitution of neutral amino acids for the acetylatable lysines in the N-terminal domains. For example, Lys→Gln substitutions at positions 5, 8 and 12 of H4 result in changes in GAL1 and PHO5 induction comparable to those seen in deletion mutants (Durrin *et al.*, 1991). The same substitution at Lys16 prevents silencing of the mating type genes (Johnson *et al.*, 1992). The Lys→Gln substitution mimics, to a first approximation, the changes brought about by lysine acetylation (i.e. loss of a unit of positive charge) and provides evidence, albeit indirect, that acetylation of specific lysine residues on particular histones plays a role in both silencing and regulation of inducible genes.

In order to test directly the role of acetylation of a specific histone at particular lysines we have produced a panel of polyclonal antisera that can distinguish H4 isoforms acetylated at one or another of the four acetylatable lysine residues (Turner and Fellows, 1989; Turner *et al.*, 1989). Using indirect immunofluorescence microscopy we have shown that these isoforms have their own characteristic distribution patterns along the interphase polytene chromosomes of *Drosophila* larvae (Turner *et al.*, 1992). Antisera to H4 acetylated at Lys5 and/or Lys8 (H4.Ac5 and H4.Ac8) label overlapping, but non-identical, islands throughout the euchromatic chromosome arms, whereas the chromocentre (consisting predominantly of β heterochromatin) is labelled weakly. In contrast, the chromocentre labels relatively strongly with antibodies to H4.Ac12. Of particular interest is the finding that the X chromosome in male larvae is strongly labelled by antibodies to H4.Ac16, whereas the male autosomes and all chromosomes in female *Drosophila* cells label only weakly. The significance of this lies in the fact that in *Drosophila* dosage compensation between male (XY) and female (XX) cells is achieved by doubling the transcriptional activity of genes on the single male X, i.e. the male X chromosome is transcriptionally hyperactive. Interestingly, although dosage compensation in mammals is achieved

by a very different mechanism (i.e. almost complete inactivation of one of the two female X chromosomes), H4 acetylation still appears to play a role, with antisera to acetylated H4 giving only very weak labelling of the inactive female X (Xi) in metaphase cells (Jeppessen and Turner, 1993). Thus acetylation of histone H4 seems to have a role in marking functional chromatin domains and, in yeast and *Drosophila* at least, the lysines that are acetylated are functionally distinct.

While immunofluorescence microscopy has provided useful information on the distribution of acetylated H4 along metaphase chromosomes, it cannot address directly (at least in mammalian cells) H4 acetylation status at the single gene level. To do this we have immunoprecipitated chromatin from human cultured HL-60 cells with site-specific antisera to acetylated H4 and analysed the distribution of specific DNAs between the antibody-bound (acetylated) and unbound fractions. The results presented here demonstrate that H4 associated with both centric and telomeric heterochromatin is essentially unacetylated, while H4 associated with several coding DNAs shows a level of acetylation comparable with that in bulk chromatin. Most importantly, we find no correlation between the level of H4 acetylation and transcriptional activity at any of the gene loci tested. We have, however, noted transient increases in the acetylation of H4 in heterochromatin coincident with differentiation along either the granulocytic or monocytic pathways. We conclude that in human cells H4 acetylation does not discriminate, in general, between genes that are transcriptionally active or inactive, but serves instead as a relatively long-term marker that distinguishes regions of the genome enriched or depleted in coding DNA.

Results

In HL-60 cells the monoacetylated isoform of H4 (H4.Ac_1) accounts for ~20% of total H4 [based on Coomassie Blue staining of acid/urea/Triton (AUT) gels] and the more highly acetylated isoforms (H4.Ac_{2-4}) between 6 and 12%. Among these, the tri- and tetra-acetylated isoforms are always rare (Turner and Fellows, 1989; see also Figure 10). In HL-60 cells, as in all mammalian cells tested so far, the order in which H4 is acetylated is non-random, with acetylation occurring at Lys16 first, followed by Lys8 or Lys12 and finally Lys5 (Turner *et al.*, 1989; Thorne *et al.*, 1990). In an attempt to take account of this pattern of lysine acetylation in trying to unravel the role of the different acetylated isoforms in transcription we have immunoprecipitated chromatin fragments with either: (i) a cocktail of antibodies to H4 acetylated at Lys5, Lys8 and/or Lys12, a mixture that should precipitate nucleosomes containing H4.Ac_{2-4}, but leave those containing only H4.Ac_0 and H4.Ac_1 in the supernatant or (ii) each individual site-specific antibody. The latter should precipitate either nucleosomes containing any acetylated H4 isoform (antiserum R14/16), those containing H4.Ac_{2-4} (R12/8 and R20/12) or just those containing the most highly acetylated isoforms, H4.Ac_{3-4} (R6/5 or R41/5).

Validation of the immunoprecipitation approach

Chromatin was prepared by mild micrococcal nuclease digestion of purified nuclei and analysed by agarose gel electrophoresis (Figure 1). Typical oligonucleosome ladders were observed in both the first supernatant (S1) and the soluble fraction (S2, see Materials and methods). To test for the presence of transcriptionally active DNA in digested chromatin we hybridized a Southern blot with a probe to the constitutively active c-*myc* gene. Figure 1B shows that c-*myc* DNA is present in both the S1 and S2 chromatin fractions, though not in the residual nuclear pellet, and in both mononucleosomes and oligonucleosomes. The intensity of the hybridization signal generally reflects the amount of DNA (i.e. the intensity of ethidium staining), indicating that there has been little or no selective destruction of c-*myc* DNA under the digestion conditions we have used. In order to make the chromatin used for immunoprecipitation (the input fraction) as representative as possible, we routinely combined S1 and S2, which together represent ~90% of the starting DNA.

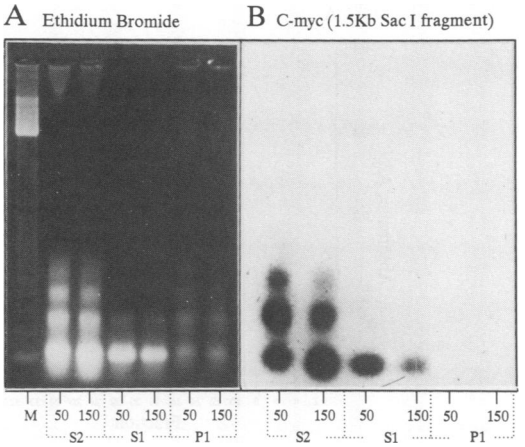

Fig. 1. Analysis of chromatin isolated from unfixed HL-60 cells. Chromatin was isolated from purified HL-60 nuclei by micrococcal nuclease digestion (50 or 150 U) at 37°C for 5 min. (**A**) The distribution of DNA (3 μg/track) between the first supernatant (S1), soluble (S2) and insoluble (P1) fractions, resolved on a 1.2% agarose gel and stained with ethidium bromide. M is a 123 bp marker (Gibco BRL). (**B**) A Southern blot of the same gel labelled with a DNA probe from the constitutively active c-*myc* gene (1.5 kb *Sac*I fragment) which encodes the first protein coding region.

Immunoprecipitation of acetylated H4 from purified mononucleosomes

Mononucleosomes were purified from sucrose gradients (Figure 2A) and peak fractions analysed by agarose gel electrophoresis. Figure 2B shows a typical mononucleosome preparation stained with ethidium bromide, followed by Southern blotting and labelling with a probe to the c-*myc* gene (Figure 2C). These results confirm that the transcripionally active c-*myc* gene is not preferentially lost during fractionation. In the following experiment a cocktail of antibodies to highly acetylated H4 was pre-incubated with mononucleosomes overnight. Immunocomplexes were isolated following addition of protein A–Sepharose and a series of high volume salt washes were performed to remove non-specifically bound material. Antibody-bound material was eluted from the protein A–Sepharose using 1% SDS and DNA and protein isolated. Equal amounts of DNA (based on [^{3}H]thymidine incorporation) from input, unbound and bound fractions were then serially diluted, loaded in duplicate onto Hybond N+

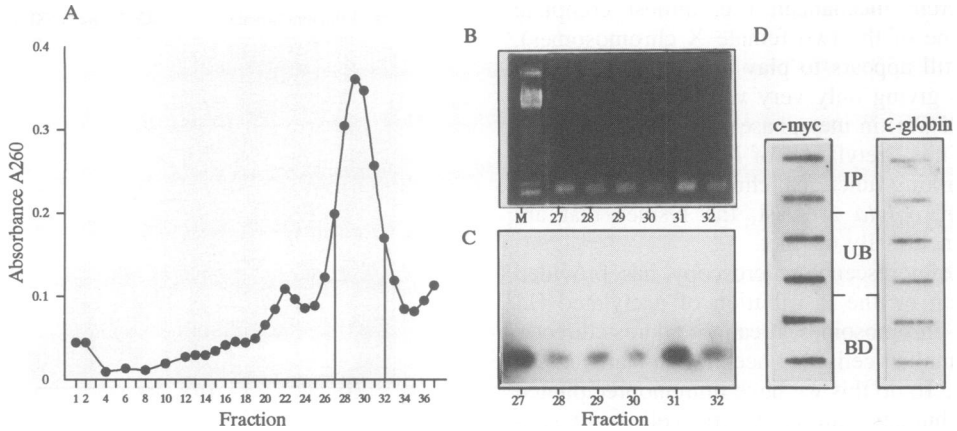

Fig. 2. Analysis of mononucleosomes following fractionation and immunoprecipitation. (**A**) A typical mononucleosome profile after centrifugation of chromatin through a 5–20% exponential sucrose gradient. The major peak (fractions 27–32) is mononucleosomes. (**B**) The size of the DNA fragments isolated from the fractions indicated and analysed in 1.2% agarose gels stained with ethidium bromide. (**C**) A Southern blot of the same gel labelled with c-*myc* (1.5 kb *Sac*I fragment). M is a 123 bp marker (Gibco BRL). (**D**) A slot-blot of DNA isolated from input (IP) unbound (UB) and bound (BD) samples. Equal amounts of DNA (based on [^{3}H]thymidine incorporation) were loaded for each fraction and five serial doubling dilutions performed, only one of which is shown here. The same filter was probed with the transcriptionally active c-*myc* (1.5 kb *Sac*I fragment) and inactive ε-globin (3.4 kb *Bam*HI–*Eco*RI fragment) gene probes.

filters and hybridized with DNA probes to transcriptionally active (c-*myc*) and inactive (ε-globin) genes. The same filter was stripped and used in each hybridization, so the results are directly comparable. Figure 2D shows that both the c-*myc* and ε-globin probes labelled the unbound and bound fractions with equal intensity. These results suggest that the level of highly acetylated H4 nucleosomes along these two genes is approximately the same, irrespective of transcriptional activity.

The same results were obtained in a number of independent experiments with purified mononucleosomes. However, these experiments inevitably use only a selected fraction of chromatin. In the example shown in Figure 2 the mononucleosome fraction accounted for 17% of total, soluble, nuclease-digested chromatin. Of this, 60% was recovered after immunoprecipitation, of which 12% was in the bound fraction. The small amount of material this represented, while adequate for DNA analysis, did not allow accurate determination of the protein composition of the bound fraction. In order to address these matters, we went on to study the distribution of highly acetylated H4 in longer oligonucleosome fragments.

Immunoprecipitation using oligonucleosome fragments

An estimate of the efficiency of the immunoprecipitation procedure after using oligonucleosome fragments as the input material was made by determining the amount of [^{3}H]thymidine-labelled DNA in the unbound and antibody-bound fractions. Table I shows the percentage of recovered DNA in the bound fraction (recovery was consistently 50–60% of input) after using either a cocktail of antisera to highly acetylated H4 or the individual site-specific antisera. With the latter the amount of DNA in the bound fraction is consistent with the frequency with which these sites are acetylated *in vivo*. For example, H4 Lys16 is the most frequently acetylated residue in HL-60 cells and this is reflected in the amount of bound material (20% of recovered DNA). In comparison, H4 acetylated at Lys5 is present predominantly (though not exclusively) in the rare

Table I. Amount of DNA precipitated by the antisera determined by [^{3}H]thymidine counts

Antibody	Recovered material precipitated (%)	No. of experiments
R14/16	20 ± 8	5
R5/12	10 ± 4.5	3
R12/8	14 ± 6.9	10
R41/5	2 ± 0.5	3
Cocktail[a]	18 ± 4	11
Pre-immune	0.3 ± 0.15	2
No antibody	0.5 ± 0.2	17

Typically the recovered material (i.e. unbound + bound) represented 50–60% of the input material. The percentage of the antibody-bound fraction from the recovered material is shown.
[a]The cocktail contains a mixture of R12/8, R5/12 and R41/5.

tetra-acetylated H4 isoform and use of an antibody specific for this site routinely results in only ~2% of recovered DNA in the bound fraction. The fact that these recoveries correspond so closely to what would be expected on the basis of the known pattern of H4 acetylation in HL60 cells argues strongly that there has been no selective loss of acetylated chromatin from the bound fraction during preparation. Non-specific binding of chromatin to the protein A–Sepharose beads, with or without pre-immune antibodies, was very low (Table I).

To check the specificity of the antibodies during the immunoprecipitation procedure proteins were analysed initially by electrophoresis in SDS-containing polyacrylamide gels (SDS–PAGE), Western blotted and labelled with the site-specific antisera. Figure 3 shows typical results following immunoprecipitation using the cocktail of antisera to highly acetylated H4. The unbound fraction was almost completely depleted of H4 acetylated at Lys5, Lys8 and Lys12 (Figure 3B). However, when an identical filter was labelled with an antibody to H4.Ac16, an equal signal was obtained from both the unbound and bound fractions. This result is entirely consistent with the order in which H4 lysines are acetylated in HL-60 cells. A

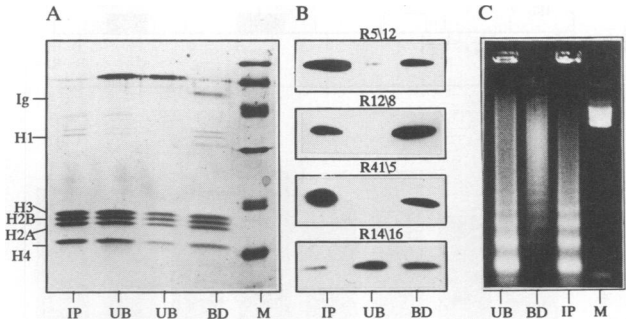

Fig. 3. Analysis of proteins and DNA following immunoprecipitation. (**A**) Proteins were isolated from input (IP), unbound (UB) and bound (BD) fractions following immunoprecipitation with a cocktail of antibodies to highly acetylated H4 (R12/8, R5/12 and R41/5). Core histones were resolved by 15% SDS–PAGE and stained with Coomassie Blue. Each track was scanned and the amount of H4 in each sample calculated. (**B**) Equal amounts of H4 (calculated as described in A) from input (IP), unbound (UB) and bound (BD) fractions were resolved by 15% SDS–PAGE, Western blotted and immunostained with either R5/12, R12/8, R41/5 (all present in the cocktail) or R14/16. Immunocomplexes were visualized using enhanced chemiluminescence (Amersham). (**C**) The size distribution of DNA isolated from input, unbound and bound fractions resolved on a 1.2% agarose gel stained with ethidium bromide.

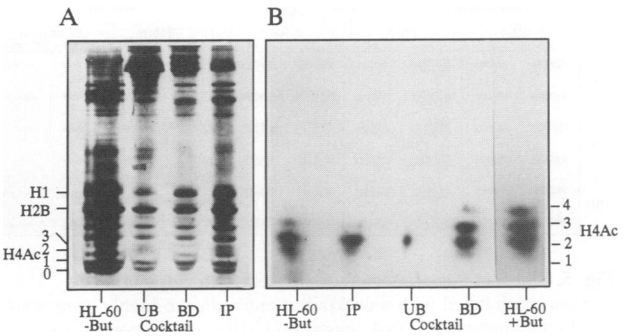

Fig. 4. Analysis of proteins on AUT gels. Proteins isolated from input (IP) unbound (UB) and bound (BD) fractions were concentrated and analysed on AUT gels. (**A**) A silver stained gel and (**B**) a second gel labelled with an antibody to H4 acetylated at Lys12 (R20/12), which recognizes the more highly acetylated H4 isoforms (i.e. $H4Ac_{2-4}$). Histones isolated from HL-60 cells untreated or treated with 5 mM sodium butyrate for 6 h were run in parallel as markers.

substantial proportion of mono-acetylated H4 (the most common isoform) is acetylated only at Lys16 and should therefore remain in the unbound fraction. On the other hand, most H4 molecules acetylated at Lys5, Lys8 or Lys12, which are recognized by the cocktail and present in the bound fraction, will be acetylated at Lys16 also and so will be recognized by R14/16 on Western blots. These protein results confirm that the antibody cocktail selectively discriminates between highly acetylated and the mono-acetylated H4 isoforms. They also show that at the level of antibody and chromatin selected depletion of highly acetylated H4 from the unbound fraction is essentially complete. This was the case for every immunoprecipitation experiment presented here.

DNA from input, unbound and bound fractions was analysed by agarose gel electrophoresis (Figure 3C). As expected, the unbound material reflected the size distribution of the input chromatin fragments. However, the bound fraction was consistently depleted in (though not devoid of) mononucleosome sized DNA and enriched in higher oligomers. Preferential immunoprecipitation of larger chromatin fragments has been observed by others (Mendelson *et al.*, 1986; Hebbes *et al.*, 1988) and is due, at least in part, to the increased probability that longer chromatin fragments will contain the epitope of interest. However, probability calculations (not shown) do not explain the magnitude of the size difference and the results suggest that acetylated H4 is preferentially found in higher oligomers after micrococcal nuclease cutting.

By diluting the bound fraction and reconcentrating it through micropore filters in order to reduce the concentration of SDS we were able to analyse its protein content by electrophoresis on AUT gels. (The input and unbound fractions were also treated in the same way; see Materials and methods.) Figure 4 shows an AUT gel stained with silver. H4 in the input and unbound fractions consisted exclusively of $H4.Ac_0$ and $H4.Ac_1$ in approximately equal proportions, whereas in the bound fraction $H4.Ac_2$ and $H4.Ac_3$ are clearly visible ($H4.Ac_4$ is obscured by another protein) and $H4.Ac_1$ is more intensely stained than $H4.Ac_0$. The presence of non-acetylated and mono-acetylated H4 in the bound fraction shows that the oligonucleosomes precipitated by the antibody cocktail do not consist of runs of nucleosomes all containing only hyperacetylated H4, but that nucleosomes containing hyperacetylated H4 are interspersed with those containing the non-acetylated or mono-acetylated isoforms.

A Western blot (Figure 4B) of the same gel labelled with R20/12 (an antibody which preferentially recognizes $H4.Ac_{2-4}$) confirms the enrichment of acetylated H4 in the bound fraction. Only a trace of $H4.Ac_2$ is seen in the unbound fraction, but there is clear staining of $H4.Ac_{2-4}$ in the bound fraction. We also note that the acetylated isoforms of the other core histones are not highly enriched in the bound fraction. For example H2B, which like H4 can be acetylated at four lysines, is not hyperacetylated in the bound fraction, though there is some enrichment in the mono- and di-acetylated isoforms compared with the unbound material (Figure 4A). More striking is the enrichment of H1 in the bound relative to the unbound fraction. This is clearly visible both on AUT gels (Figure 4A) and using SDS–PAGE (Figure 3A). (Note that the apparent increase in the amount of H1 relative to core histones in Figure 4 compared with Figure 3 is due to the use of silver stain rather than Coomassie Blue. Identification of the enriched band as H1 was confirmed by comparing its mobility with that of purified H1.) Enrichment of the bound fraction in H1 is consistent with the larger size of the oligonucleosomes in this fraction and raises the interesting possibility that the relative resistance of nucleosomes containing hyperacetylated H4 to micrococcal nuclease digestion is due to its effect on the interaction between H4 and H1. This is being investigated. It is important to note that the precipitation of nucleosomes containing hyperacetylated H4 by these antisera is not dependent on the presence of H1. It occurs with both purified mononucleosomes devoid of H1 (Figure 2) and in the presence of 0.4 M NaCl, in which case the H1 remains exclusively in the unbound fraction (not shown).

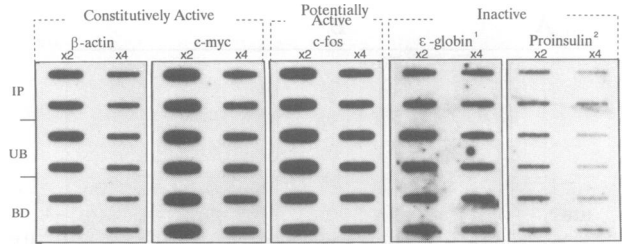

Fig. 5. Slot-blot analysis of DNA isolated from chromatin immunoprecipitated with a cocktail of antibodies to highly acetylated H4. Equal amounts of DNA (based on [^{3}H]thymidine counts) from input (IP), unbound (UB) and bound (BD) fractions were loaded in duplicate onto Hybond N+. Five serial doubling dilutions were loaded, only two of which are shown here (×2 and ×4). The same filter was used in each of the following labellings and complete stripping of each probe was determined by overnight autoradiography. DNA probes are as follows: constitutively active, β-actin 1.1 kb *Pst*I fragment and c-*myc* 1.7 kb *Pst*I fragment; potentially active, c-*fos* 3.4 kb *Bam*HI–*Eco*RI fragment; inactive[1], ε-globin 1.3 kb *Bam*HI–*Eco*RI (this gene is active elsewhere in the haemopoetic lineage) fragment; inactive[2], proinsulin 500 bp *Eco*RI fragment (this gene is never active within the haemopoetic lineage).

H4 acetylation and transcriptional activity

Equal amounts of DNA (based on [^{3}H]thymidine incorporation) from input, unbound and bound fractions were serially diluted, loaded in duplicate onto Hybond N+ filters and hybridized with DNA probes of differing sizes derived from genes with varying levels of transcriptional activity. The same filters were stripped and used for hybridization with several probes, so results with different probes are directly comparable. As shown in Figure 5, probes to all coding DNAs, irrespective of their transcriptional status, labelled the input, unbound and bound fractions with approximately equal intensity. This has been a consistent finding in numerous independent experiments with a variety of probes. The results suggest that the level of H4 acetylation in the genes tested varies little from that in bulk chromatin. The only difference from this pattern was seen with probes to DNA sequences typical of centric and telomeric heterochromatin, which, as discussed in more detail later, were found almost exclusively in the unbound fraction.

H4 acetylation along the c-myc gene

It is possible that the results presented in Figure 5 are due to relatively high levels of H4 acetylation occurring only at particular sites along these genes. Relatively large probes (i.e. equivalent to several nucleosomes) will inevitably detect DNA associated with both acetylated and adjacent non-acetylated nucleosomes, the former in the bound and the latter in the unbound fraction. To test for acetylation of positioned nucleosomes or clusters of nucleosomes we prepared probes to adjacent regions along the c-*myc* gene and used these for hybridization to bound and unbound fractions. As shown in Figure 6, there were only minor variations in the level of H4 acetylation along this gene. Further experiments with oligonucleotide probes (30mers) to sites known to contain positioned nucleosomes have given similar results (not shown). The same results were obtained when immunoprecipitation was with a lysine-specific antibody (R12/8, as shown in Figure 6) or with the antibody cocktail (not shown). Thus there is no

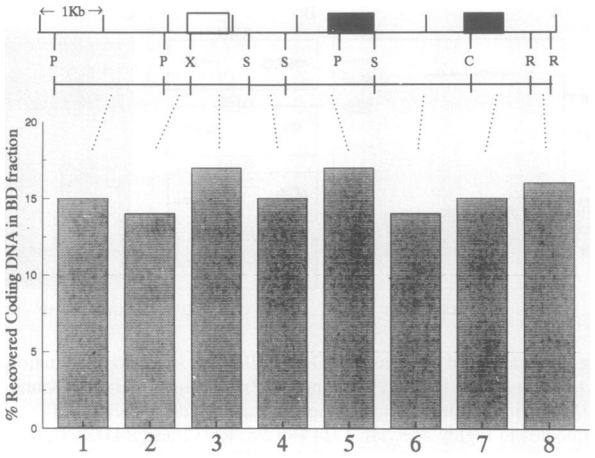

Fig. 6. The distribution of nucleosomes containing highly acetylated H4 along the constitutively active c-*myc* gene. Following immunoprecipitation of chromatin from untreated HL-60 cells with an antibody to H4.Ac8 (R12/8), the distribution of nucleosomes containing highly acetylated H4 along the c-*myc* gene was examined using various DNA probes (numbered 1–8), obtained by restriction enzyme digestion. In this experiment R12/8 precipitated 14% of the recovered material. A histogram showing the percentage recovered coding DNA in the bound fraction for each probe labelling is shown. Similar results have been obtained after using R41/5, R5/12 or a cocktail of antisera in the immunoprecipitation experiments. Probes 1–8 are as follows: 1, 1.7 kb *Pst*I–*Pst*I; 2, 480 bp *Pst*I–*Xho*I; 3, 870 bp *Xba*I–*Sac*I; 4, 606 bp *Sac*I; 5, 1.5 kb *Sac*I; 6, 1.2 kb *Sac*I–*Cla*I; 7, 879 bp *Cla*I–*Rsa*I; 8, 400 bp *Rsa*I.

evidence for increased levels of H4 acetylation at particular sites along the c-*myc* gene.

The role of acetylation at specific lysine residues

To determine whether H4 isoforms acetylated at particular lysines have different genomic distributions in HL-60 cells we immunoprecipitated chromatin with each of the site-specific antisera. Results from a single representative experiment are shown in Table II. For each antiserum the proportion of recovered coding DNA in the bound fraction corresponded closely to the proportion of total input DNA in this fraction, i.e. there was no evidence of any selective increase or decrease in the level of acetylation at any one of the four acetylatable lysines on coding DNA, irrespective of transcriptional status.

Induced changes in transcriptional activity and the distribution of acetylated H4

Addition of various inducers of differentiation to HL-60 cells leads to a change in the transcriptional activity of the proto-oncogenes c-*myc* and c-*fos*. Growth in 1.25% dimethylsulfoxide (DMSO) induces terminal differentiation along the granulocytic pathway over a period of 72 h (Collins *et al.*, 1978). During this time RNA polymerase II stalls at the P2 promoter (Strobl and Eick, 1991; Krumm *et al.*, 1992) and the level of c-*myc* mRNA decreases. Changes in chromatin structure, as determined by mapping of DNase I-hypersensitive sites, have also been observed along the c-*myc* gene after DMSO treatment (Siebenlist *et al.*, 1988). Conversely, if HL-60 cells are treated with phorbol esters such as TPA they differentiate into monocyte-like cells (Collins *et al.*, 1978). Once again, c-*myc* is down-regulated, but there is a rapid increase in the transcriptional activity of the normally quiescent c-*fos*

Table II.

Antibody	Recovered material precipitated into the bound fraction[a] (%)	Recovered gene sequence in the bound fraction (%)								
		Coding sequences						Non-coding sequences		
		Constitutively active		Potentially active	Inactive[b]		Inactive[c]	Inactive		
		β-Actin	c-*myc*	c-*fos*	ε-Globin	*RAG*-1[d]	Proinsulin	HeT266	Het405	Telomeric
R14/16	30	31	30	29	29	30	1		1	
R5/12	7	7	8	7	6	6	7	0.5	3	
R12/8	16	15	16	15	16	15	16	0.2	1	1
R41/5	2	2	3	3	2		2	0.2	0.5	
Cocktail	22	21	22	19	20	21	20	0	0.6	0.5

[a]Results are taken from one typical experiment.
[b]Inactive genes within HL-60 cells but active within the haemopoetic cell lineage.
[c]Inactive gene within HL-60 cells and the haemopoetic cell lineage.
[d]*RAG*-1 is the recombination activating gene (Schatz *et al.*, 1989).

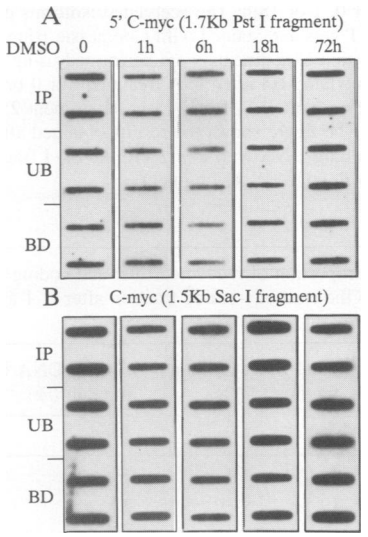

Fig. 7. Level of acetylated H4 on c-*myc* DNA following down-regulation with DMSO. HL-60 cells were treated with 1.25% DMSO for 0, 1, 6, 18 or 72 h. Chromatin isolated at each time point was immunoprecipitated with a cocktail of antisera (R12/8, R5/12, R41/5) and equal amounts of DNA from input (IP), unbound (UB) and bound (BD) fractions loaded in duplicate onto Hybond N+ (only one dilution from a series of five doubling dilutions is shown). The same filters were probed with (**A**) a 1.7 kb *Pst*I fragment from the 5′ region of the c-*myc* gene, which remains transcriptionally active following DMSO treatment, and (**B**) a 1.5 kb *Sac*I fragment which encodes the first protein coding region of c-*myc*, which is down-regulated following DMSO treatment (Siebenlist *et al.*, 1988; Strobl and Eick, 1991; Krumm *et al.*, 1992).

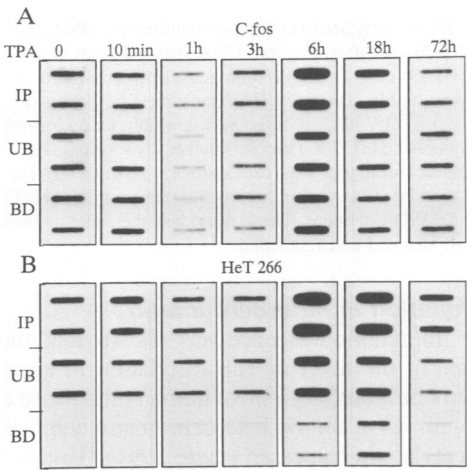

Fig. 8. Level of acetylated H4 on c-*fos* and simple satellite repeat DNA following treatment with TPA. HL-60 cells were treated with 5×10^{-7} M TPA for 0 or 10 min or 1, 3, 6, 18 or 72 h. Chromatin isolated at each time point was immunoprecipitated with a cocktail of antibodies to highly acetylated H4. Equal amounts of DNA from input (IP), unbound (UB) and bound (BD) fractions were loaded in duplicate onto Hybond N+. (**A**) The distribution of the normally quiescent c-*fos* gene (a 3.4 kb *Bam*HI–*Eco*RI fragment), which is rapidly induced following TPA treatment (Muller *et al.*, 1985). Similar distributions of the constitutively active c-*myc* gene and the inactive ε-globin gene were also observed (results not shown). (**B**) The distribution of a simple satellite III repeat (HeT266) characteristic of centric heterochromatin. The same filters were stripped and used in each labelling and the results are therefore directly comparable.

gene (Muller *et al.*, 1984). Addition of these inducers allows us to ask whether a change in the transcriptional activity of a gene is reflected in changes in the acetylation status of H4.

Chromatin was prepared from HL-60 cells after growth in 1.25% DMSO for 0, 1, 6, 18 and 72 h and immunoprecipitated with a cocktail of antibodies to acetylated H4. DNA slot-blots were labelled with probes to various regions along the c-*myc* gene, only two of which are shown in Figure 7. The first c-*myc* probe shown corresponds to a region 5′ of the c-*myc* P2 promoter, which is still transcribed after differentiation, whereas the second probe detects a 3′ region which is not transcribed following DMSO treatment. (Northern blot analysis of c-*myc* RNA confirmed that DMSO had effectively down-regulated c-*myc*; results not shown.) DNA from the bound and unbound fractions labelled with approximately equal intensity with both probes, irrespective of DMSO treatment.

We also prepared and immunoprecipitated chromatin from HL-60 cells treated with TPA in order to induce the c-*fos* gene. c-*fos* expression was rapidly induced, reaching a maximum after 15–30 min (not shown). Using a probe covering both the promoter and coding regions no TPA-induced changes were found in the distribution of c-*fos* DNA between the bound and unbound fractions (Figure 8A).

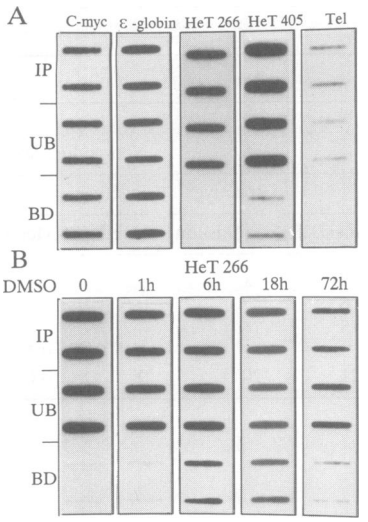

Fig. 9. Level of acetylated H4 on heterochromatin DNA and the effect of DMSO-induced differentiation. DNA isolated from chromatin from untreated HL-60 cells following immunoprecipitation with a cocktail of antisera to highly acetylated H4. The same filter was used for each labelling. c-*myc* (1.7 kb *Pst*I fragment), ε-globin (1.2 kb *Bam*HI–*Eco*RI fragment), HeT 266 (simple satellite III repeats), HeT 405 (alphoid heterochromatin) and Tel (telomeric repeat). (**B**) The change in highly acetylated H4 within heterochromatic regions (HeT266) following DMSO treatment. These filters are the same as those shown in Figure 7, stripped and relabelled.

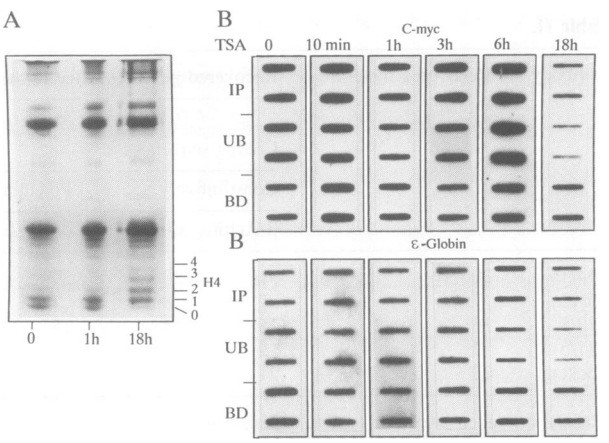

Fig. 10. Effect of trichostatin A on the level of acetylated H4 on c-*myc* and ε-globin DNA. (**A**) Histones were isolated from HL-60 cells following treatment with trichostatin A (an inhibitor of histone deacetylases) for 0, 1 or 18 h. The acetylated isoforms of H4 were resolved on AUT gels and stained with Coomassie Blue. (**B**) Two immunoprecipitation experiments were performed using a cocktail of antibodies to acetylated H4 after TSA treatment for 0 or 10 min or 1, 3, 6 or 18 h (experiment 1: 0, 1 and 18 h; experiment 2: 0 and 10 min and 3 and 6 h). The figure shows the results obtained after slot-blotting and labelling DNA with c-*myc* (1.5 kb *Sac*I fragment) or ε-globin (1.2 kb *Bam*HI–*Eco*RI fragment).

H4 acetylation in heterochromatin

Indirect immunofluorescence has shown that there is a difference in the level of H4 acetylation in euchromatic and heterochromatic regions of human metaphase chromosomes, with H4 in centric heterochromatin being relatively under acetylated (Jeppessen *et al.*, 1991). To test whether this is also true in interphase cells we used DNA probes to simple satellite DNA repeats, characteristic of constitutive centric and telomeric heterochromatin, and analysed their distribution between the bound and unbound fractions. All these sequences were found to be either absent or under-represented in the bound fraction (Figure 9A). In view of the selective acetylation of heterochromatin H4 at Lys12 in *Drosophila* (Turner *et al.*, 1992), it was of interest to test the level of acetylation at specific lysines in mammalian heterochromatin. Precipitation with site-specific antibodies showed that H4 in centric heterochromatin in human HL-60 cells is under-acetylated at all four acetylatable lysines (Table II).

Turnover of H4 acetates along transcriptionally active and inactive genes

In order to determine whether the rate of H4 acetylation along active and inactive genes differed, HL-60 cells were treated with the deacetylase inhibitor trichostatin A (TSA) for varying lengths of time (0–18 h) before isolation of chromatin and immunoprecipitation. If nucleosomes associated with transcriptionally active genes show a high rate of acetate turnover, then inhibition of the deacetylating enzymes should shift the actively transcribed gene sequences into the bound (i.e. acetylated) fraction. The rate at which this shift occurs will reflect the rate at which acetate groups are turning over.

Growth in TSA resulted in the expected increase in acetylation of bulk histones resolved by electrophoresis in AUT gels (Figure 10A). There was also an increase in the amount of material in the bound fractions after immunoprecipitation, exactly as expected (Table III). Figure 10B shows the results obtained after labelling the same filter with a probe to either c-*myc* or ε-globin. Growth of cells in TSA for up to 6 h caused a slight preferential increase in the level of acetylation of H4 associated with these genes (Table III). Similar results were obtained with probes to actin or c-*fos* (not shown) and we conclude that the acetate groups on H4 associated with these coding DNAs turn over slightly faster than those in bulk chromatin. More importantly, we find no differences related to the transcriptional status of the gene. Acetates on the transcriptionally active c-*myc* and the quiescent ε-globin genes turn over at a similar rate (Table III). There was a clear increase in the proportion of coding DNA in the bound fraction after 18 h growth in TSA (Figure 10 and Table III), showing that at this stage there had been an increase in the acetylation of H4 in coding regions over and above that which occurs in bulk chromatin. Whether this was due to subtle differences in turnover of H4 acetates or to the differentiation-inducing effects of TSA over longer times remains to be established. Once again, there was no difference between transcrip-

Table III. The proportion of recovered total and coding DNA precipitated into the antibody-bound fraction after 0, 1 and 18 h growth in TSA

Time in TSA (h)	Total DNA in bound fraction (%)	Coding DNA in bound fraction (%)	
		c-*myc*	ε-Globin
0	15	17	16
1	19	22	23
18	25	34	33

tionally active and quiescent genes in the level of H4 acetylation.

Differentiation-induced changes in acetylated H4 in heterochromatin

When we examined the distribution of highly acetylated H4 within constitutive heterochromatin during differentiation along the granulocytic pathway an unexpected change was found. Figure 9B shows the distribution of a simple satellite III sequence following DMSO treatment. In untreated HL-60 cells the bound fraction (highly acetylated) was depleted in heterochromatin sequences, as observed earlier. However, during DMSO treatment a transient shift of these sequences into the bound fraction occurred. This was most pronounced after 6–18 h (Figure 9B). After prolonged DMSO treatment (72 h), when the cells had entered a terminally differentiated state, these regions once again become depleted in highly acetylated H4. This transient change in the acetylation status of H4 in heterochromatin was reproducible in several independent experiments with different antisera (the cocktail and R12/8). A similar transient shift was noted during TPA-induced differentiation down the monocytic pathway (Figure 8B).

Discussion

A link between histone acetylation and transcription was first proposed by Allfrey (1964) and in more recent years a range of indirect evidence has accumulated to support this suggestion. Chromatin fractions enriched in transcribing DNA have been prepared by a variety of techniques, including limited nuclease digestion (Weintraub and Groudine, 1976; reviewed by Mathis *et al.*, 1980), differential solubility in Mg^{2+}-containing buffers (Perry and Chalkley, 1981, and references therein), mercury affinity chromatography (Allegra *et al.*, 1987; Chen and Allfrey, 1987; Chen *et al.*, 1990), density gradient sedimentation (Ip *et al.*, 1989) and isolation of CpG islands (Tazi and Bird, 1990). These fractions have often been found to be enriched (though sometimes only modestly) in acetylated core histones. However, even in those cases where the level of histone acetylation is clearly increased, highly acetylated histones may not be located on the transcribing DNA itself. They may simply co-purify, possibly because the chromatin on which they are located shares common properties with transcriptionally active chromatin.

Direct experimental evidence linking histone acetylation and transcription at the single gene level has been obtained by immunoprecipitation of chromatin fragments with an antibody that recognizes ε-acetyllysine. The antibody-bound fraction derived from chicken erythrocyte chromatin was shown to be enriched in both highly acetylated core histones and the transcriptionally active β-globin gene. It was not enriched in the inactive ovalbumin gene (Hebbes *et al.*, 1988). The β-globin gene was also associated with relatively highly acetylated core histones in erythrocytes from 5 day embryos, at which developmental stage it is not expressed, showing that, at least in the erythroid cell lineage, both transcriptionally active and potentially active genes carry a similar level of highly acetylated core histones. More recently, core histone hyperacetylation along the β-globin locus has been shown to coincide with the entire DNase I-sensitive domain, whereas only low levels of hyperacetylated core histones were observed at the boundary (Hebbes *et al.*, 1994). Thus acetylation is not simply a consequence of transcription, but may render a gene locus transcriptionally competent in a heritable fashion (Hebbes *et al.*, 1992, 1994; Clayton *et al.*, 1993). One limitation of these experiments is that the broad specificity of the antibody used prevents any distinction being made between acetylation of different core histones or between acetylation of different lysines on the same histone. In some species at least, both of these factors seem to be functionally significant (reviewed in Turner, 1993).

Braunstein *et al.* (1993) used an antibody to *Tetrahymena* acetylated H4 to immunoprecipitate chromatin from the yeast *Saccharomyces cerevisiae* in order to determine the acetylation status of H4 along transcriptionally silent or active mating type cassettes. They showed that silencing of these regions was accompanied by under-acetylation of the associated H4 and that this H4 was acetylated in strains in which the mating type cassettes were transcriptionally active. However, they also showed that acetylation occurred even in promoter-deficient strains in which the gene could not actually be transcribed. Thus, in this situation at least, H4 acetylation seems to be involved in the prevention of silencing, rather than in the transcriptional process *per se*.

In the experiments described here we have used an immunoprecipitation approach to define the level of acetylation of histone H4 within specific genes and to assay for variation in H4 acetylation corresponding to both constitutive and induced differences in transcriptional activity. Using a panel of site-specific antisera we have been able to look for functionally related differences in acetylation at specific H4 lysine residues. The results may be summarized as follows.

(i) Histone H4 within or adjacent to coding regions of the genome showed a level of acetylation equal to or only slightly above that found in the input chromatin. The frequency with which individual lysines were acetylated in H4 associated with coding regions generally reflected that in input chromatin (i.e. $16 > 8/12 > 5$).

(ii) H4 associated with centric heterochromatin or with the CCCTAA repeat of telomeric heterochromatin was infrequently acetylated (<1%) at all lysines.

(iii) The acetylation of H4 in coding DNA did not vary to any major extent with transcriptional activity and, at least in the case of the constitutively active c-*myc* gene, did not vary with position along the gene.

(iv) The level of H4 acetylation in coding regions was slightly increased, relative to that in bulk chromatin, by growth of cells in an inhibitor of histone deacetylation, irrespective of transcriptional status.

(v) Induction of differentiation of HL-60 cells by exposure to DMSO or TPA did not alter the level of H4 acetylation within either the c-*myc* or c-*fos* genes or other coding regions, but did induce a transient increase in H4 acetylation within centric heterochromatin.

The experiments described here were all conducted with material from cultured human HL-60 cells. In view of the unexpected nature of some of the results, it is important to note that recent findings have shown that the results are not peculiar to this cell line. Immunoprecipitation of chromatin from adult mouse liver has confirmed the under-acetylation of H4 in heterochromatin and, more

importantly, experiments with chromatin from both mouse liver and primary fetal fibroblasts confirm the absence of any general correlation between transcriptional status and the acetylation of H4 (L.P.O'Neill and B.M.Turner, unpublished results). It is also significant that experiments in which the same immunoprecipitation approach has been applied to chromatin from *Drosophila* Kc cells have shown a rapid and selective increase in H4 acetylation during induction of the HSP70 heat shock gene (R.J.L.Munks, L.P. O'Neill and B.M.Turner, unpublished results). Thus both the technique and the antibodies we have used are together capable of generating high levels of enrichment of specific sequences in antibody-bound fractions.

The distribution of acetylated H4 within and adjacent to coding regions

The possible effects of technical or experimental factors on the results presented here have been considered in the previous section. We find no evidence that selective nuclease digestion of coding regions, incomplete (or non-specific) precipitation by the antibodies, selective loss of antibody-bound chromatin, or hybridization artefacts have made any significant contribution to the results.

In the sample of genes we have tested (representing constitutively active, inducible and inactive genes) the level of H4 acetylation and the frequency with which individual lysines are acetylated are little different to the values in bulk chromatin (i.e. both seem to reflect the overall frequency of H4 acetylation). The interpretation that is most consistent with the results presented here is that there is a scattered, probably random, distribution of acetylated H4 across these coding (and adjacent) regions. The alternative explanation, namely that there are small regions within each gene with high levels of acetylated H4, is not consistent with either (i) the very similar levels of H4 acetylation in defined, adjacent regions along the c-*myc* gene, (ii) the fact that very similar results were obtained when the starting (input) chromatin was in the form of oligonucleosomes or gradient-purified mononucleosomes and (iii) the presence of $H4.Ac_0$ and $H4.Ac_1$ in the bound fraction.

In view of the findings of Ip *et al.* (1988), who used labelling with [^{3}H]acetate to show that histones in chromatin fractions enriched in transcriptionally active genes turn over their acetate groups more rapidly than histones in bulk chromatin, we have carefully considered the possibility that the distribution of coding DNA between the bound and unbound fractions reflects a dynamic equilibrium between acetylated and non-acetylated H4 isoforms in and adjacent to coding regions. In fact, our results show that while turnover of H4 acetates in coding regions as a whole is slightly above that in bulk chromatin, there is no difference between transcriptionally active and quiescent genes. The fractionation scheme used by Ip *et al.* (1988) involved the separation of formaldehyde-fixed chromatin fragments of average size 10–15 kb on caesium chloride gradients. A light fraction was found to be enriched in actively transcribed genes, RNA polymerase II and rapidly acetylated histones. The presence of genes in the light fraction was found to be dependent on the integrity of the RNA transcript and the authors point out that the DNA within this fraction must be associated with proteins other than polymerase molecules, which are themselves unlikely to be present in sufficient numbers to account for the major reduction in bouyant density. Proteins of the nuclear matrix are suggested as possible candidates. Thus the fraction contained much besides coding DNA and associated proteins and so the fact that a rapidly acetylated histone subpopulation is also present in this fraction does not necessarily mean that it is located on coding DNA itself or on closely adjacent sequences. It could, for example, be located on matrix-associated chromatin (Schlake *et al.*, 1994).

The same caveat can be applied to the results of Allfrey and co-workers, who have developed what appears to be an effective and sensitive method for isolating transcriptionally active chromatin by affinity chromatography on an organomercury column (Allegra *et al.*, 1987). The procedure depends on exposure of an H3 sulfhydryl group as a consequence of structural changes in transcriptionally engaged nucleosomes. The c-*myc* and c-*fos* genes are both bound by such columns when transcriptionally active (Chen and Allfrey, 1987; Chen *et al.*, 1990). It has also been claimed that bound nucleosomes are enriched in acetylated histones (Boffa *et al.*, 1990). However, this claim relies solely on the interpretation of H4 banding patterns after Coomassie Blue staining of AUT gels. The possibility that one or more of the many non-histone proteins present in the material eluted from the column may co-migrate with the highly acetylated H4 isoforms cannot be discounted, nor can the possibility, noted above, that acetylated nucleosomes and those associated with transcribing DNA simply co-purify.

H4 acetylation and short-term switching of transcriptional activity

The experimental approach described here was initiated in order to test the involvement of H4 acetylation in the short-term switching of gene activity, specifically the induction and suppression respectively of the proto-oncogenes c-*fos* and c-*myc*. The answer is quite clear. H4 acetylation is not involved in this process, nor does it discriminate in general terms between transcriptionally active and inactive genes. There is no requirement for high levels of H4 acetylation within either the coding or promoter regions of the constitutively active c-*myc* gene and none of the other transcriptionally active genes we have tested show any sign of an overall increase in H4 acetylation. We can conclude that ongoing transcription does not require high levels of H4 acetylation. Further, the results on the induction of c-*fos* with TPA make it unlikely that initiation of transcription requires H4 acetylation across the coding or promotor regions of the c-*fos* locus, though transient acetylation limited to one or a small number of nucleosomes within the promotor would not have been detected with the probes we have used so far.

H4 acetylation in constitutive heterochromatin and euchromatin

Our results show a sharp contrast between the level of acetylation of H4 in coding regions and that of H4 associated with simple satellite repeat DNA in both centric and telomeric heterochromatin. H4 in these regions is non-acetylated or at least acetylated infrequently. The results presented are generally consistent with deductions made from immunofluorescence microscopy of metaphase

chromosomes. Under-acetylation of H4 in centric heterochromatin has been noted in both human and mouse cells (Jeppessen *et al.*, 1991; Jeppessen and Turner, 1993). Further, the characteristic banding observed along chromosome arms after immunostaining with antisera to H4 acetylated at Lys5, Lys8, Lys12 or Lys16 has been attributed to preferential labelling of R bands (Jeppessen and Turner, 1993), regions known to be enriched in coding DNA. Thus most acetylated H4 molecules are associated with R band DNA. As R bands account for ~45% of the human genome (Craig and Bickmore, 1994), the level of H4 acetylation at any particular site within an R band domain is not necessarily particularly high or, put another way, nucleosomes containing acetylated H4 need not be particularly frequent. Nevertheless, if acetylated H4 were to be confined to R band domains then one would expect the level of H4 acetylation at loci within these domains to be about twice that of bulk chromatin. In the genes we have tested so far this is not generally the case, though particular regions can approach this level. However, it is possible, even likely, that regions yet to be identified will show a higher than average level of H4 acetylation, whereas others may show lower levels. It is also likely that DNA sequences will be found, other than the simple satellite repeats identified so far, that are usually associated with non-acetylated H4. To understand the role of H4 acetylation in genomic function it seems that it will be necessary to consider its role in the assembly and maintenance of chromosome domains well beyond the single gene level.

DMSO-induced differentiation of HL-60 cells along the granulocytic pathway caused no change in the acetylation of H4 in coding regions, but did result in a transient increase in acetylation of heterochromatin-associated H4. A differentiation process leading to a non-dividing, terminally differentiated cell type with a characteristic nuclear morphology would be expected to involve major remodelling of nuclear chromatin and the acetylation and deacetylation of heterochromatin H4 may be part of this process. As the same shift occurred during TPA-induced differentiation down the monocyte pathway, such a remodelling function is not lineage specific. Immunofluorescent labelling of metaphase chromosomes from mouse embryo stem cells shows that H4 in centric heterochromatin is acetylated in undifferentiated cells at a level comparable with that found along the chromosome arms, but becomes deacetylated as the cells differentiate in culture (A.M.Keohane and B.M.Turner, unpublished results). Also, immunoprecipitation of chromatin from mouse embryo fibroblasts has shown a level of H4 acetylation in heterochromatin comparable with that of H4 associated with coding DNA (A.Ferguson-Smith, L.P.O'Neill and B.M.Turner, unpublished results). Collectively these results suggest that the level of acetylation of H4 is more flexible in heterochromatin than in the coding regions of euchromatin. Perhaps changes in the acetylation status of H4 in heterochromatin are an integral part of changes in nuclear structure and function during differentiation and development.

Materials and methods

Antisera to acetylated H4

Polyclonal antisera to acetylated H4 were raised in rabbits by immunization with synthetic peptides corresponding to regions within the N-terminal domain of histone H4 and containing acetyllysine residues at defined positions. The preparation and characterization of these antisera have been described in detail elsewhere (Turner and Fellows, 1989; Turner *et al.*, 1989, 1992). For immunoprecipitation of chromatin fragments antibodies were affinity purified by binding to the appropriate peptide conjugated to CH-Sepharose (Pharmacia). After extensive washing of the resin, first with phosphate-buffered saline (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01% sodium azide (PPA) and then with 1 M NaCl in PPA, antibodies were eluted with 8 M urea, dialysed overnight at 4°C against several changes of PPA, mixed with an equal volume of glycerol and a 0.1 volume of 20 mg/ml bovine serum albumin (ELISA grade; Sigma) and stored at –20°C

Cultured cells

Human promyeloid HL-60 cells were grown in RPMI medium (Gibco BRL) supplemented with 8% fetal calf serum (Gibco BRL) in an atmosphere of 5% CO_2 in air. Cell densities were maintained between 1×10^5 and 1×10^6 cells/ml. DMSO (Sigma) and TPA (Sigma) were added directly to the culture medium at 1.25% and 5×10^{-7} M final concentration respectively. HL-60 cells plus inducer were grown at 37°C for the required time prior to harvesting by centrifugation. To induce histone hyperacetylation TSA (a generous gift from Dr M.Yoshida) was added to cultured HL-60 cells at 0.1 μg/ml prior to chromatin preparation and immunoprecipitation. Histones were extracted from nuclear pellets with 0.2 N HCl as previously described (Turner and Fellows, 1989) and analysed on AUT gels (Alfageme *et al.* 1974).

Preparation of chromatin from HL-60 cells

HL-60 cells were grown to a density of 10^6 cells/ml and labelled overnight with 0.5 μCi/ml [^{3}H]thymidine (Amersham). For each immunoprecipitation experiment ~2×10^8 cells were harvested by centrifugation (220 *g*, 4°C, 10 min) and washed three times in ice-cold PBS containing 5 mM sodium butyrate. Cell pellets were resuspended in 1× TBS (0.01 M Tris–HCl, pH 7.5, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium butyrate) to 2×10^7 cells/ml and an equal volume of 1% Tween 40 in TBS and a 1/200 volume 0.1 M PMSF added. After stirring on ice for 1 h cell suspensions were homogenized in an all glass homogenizer 'A' pestle with nuclei extrusion, followed by microscopy. Homogenates were centrifuged at 600 *g* for 20 min and the pellet resuspended in 25% sucrose in TBS at 10^6 nuclei/ml and underlayered with 50% sucrose in TBS. After centrifugation (1500 *g*, 4°C, 20 min) the nuclear pellet was washed once in 25% sucrose and then resuspended in digestion buffer (0.32 M sucrose, 50 mM Tris–HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, 5 mM sodium butyrate) to 0.5 mg DNA/ml (by absorbance at $\lambda_{260/280}$).

Chromatin was released from nuclear preparations by digestion at 37°C for 5 min with micrococcal nuclease (10–150 U; Pharmacia). Digestion was stopped by addition of 0.2 M EDTA to a final concentration of 5 mM and cooling on ice. The first supernatant following centrifugation (11 600 *g*, 10 min) was designated S1. The pellet was resuspended in lysis buffer (1 mM Tris–HCl, pH 7.4, 0.2 mM Na_2EDTA, 0.2 mM PMSF, 5 mM sodium butyrate) and dialysed for at least 12 h at 4°C against 2 l of the same buffer. Solubilized chromatin (S2) was clarified by centrifugation (600 *g*, 4°C, 10 min). Insoluble pelleted material (P1) was resuspended in 250 μl lysis buffer. Chromatin preparations were analysed on agarose gels (Maniatis *et al.*, 1982) to determine the extent of micrococcal nuclease digestion and S1 and S2 were routinely combined (~90% total chromatin DNA) and used immediately.

Isolation of H1-depleted mononucleosomes

Chromatin (300–400 μg) was layered onto 5–20% exponential sucrose gradients containing 0.02 M EDTA, 1 mM Tris–HCl, pH 7.4, 0.4 M NaCl, 25 mM sodium butyrate, 0.1 mM PMSF and centrifuged at 40 000 r.p.m. ($\omega^2t = 6.1\times10^{11}$) at 4°C for 22 h (Beckman SW40). Gradients were fractionated from the bottom and analysed in an LKB uvicord. The absorbances of all fractions at 260 and 280 nm were read using a spectrophotometer (Pharmacia Ultrospec III) and peak fractions of mononucleosomes pooled and used immediately. Approximately 17–20% of the input chromatin (S1 and S2 combined) is typically recovered as mononucleosomes.

Immunoprecipitation from unfixed HL-60 chromatin

The following procedure for the immunoprecipitation of acetylated H4 from unfixed HL-60 chromatin has been developed from the methods employed by Hebbes *et al.* (1988) and Kamakaka and Thomas (1990). Siliconization of Eppendorf tubes and pipettes was found to be essential for maximum recovery of DNA. Affinity-purified antibody (100–200 μl,

50–100 μg Ig) was added to 100–200 μg unfixed chromatin and the final volume made up to 1 ml with incubation buffer (50 mM NaCl, 20 mM Tris–HCl, pH 7.5, 20 mM sodium butyrate, 5 mM Na_2EDTA, 0.1 mM PMSF). After overnight incubation (on a rotating platform) at 4°C, 200 μl 50% w/v protein A–Sepharose (Pharmacia) was added and the incubation continued for a further 3 h at room temperature. After centrifugation (11 600 *g*, 10 min) the supernatant was removed and stored on ice (unbound fraction) and the protein A–Sepharose pellet was resuspended in 1 ml buffer A (50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate) containing 50 mM NaCl and layered onto 9 ml of the same buffer. Following centrifugation (600 *g*, 4°C, 10 min) the supernatant was removed by aspiration and the pellet washed firstly in 10 ml buffer A containing 100 mM NaCl and finally in 10 ml buffer A containing 150 mM NaCl. Bound material was eluted from the protein A–Sepharose by addition of 125 μl 1% SDS in incubation buffer and incubation for 15 min at room temperature on a rotating platform. After centrifugation (11 600 *g*, 10 min) the supernatant was removed and stored on ice. The protein A–Sepharose pellet was extracted as above with a further 125 μl 1% SDS in incubation buffer. The two extracts were combined with an equal volume of incubation buffer to reduce the concentration of SDS to 0.5%.

DNA was obtained from the input, unbound and bound fractions by two phenol/chloroform extractions and one chloroform extraction. DNA was ethanol precipitated using glycogen (5 μg) as a carrier and redissolved in 250 μl TE buffer (10 mM Tris–HCl, 2 mM EDTA, pH 7.4). All DNA samples were analysed initially by electrophoresis on 1.2% agarose gels and staining with ethidium bromide. [^{3}H]Thymidine in each sample was determined by scintillation counting. Southern blot analysis was carried out as described in Maniatis *et al.* (1982).

Proteins from input, unbound and bound fractions were obtained from the first phenol/chlorform phase (Matthews *et al.*, 1979) by addition of 5 μg bovine serum albumin (carrier), a 1/100 volume of 10 M H_2SO_4 and 12 volumes of acetone. After overnight precipitation at –20°C the protein pellets were washed once in acidified acetone (1:6 100 mM H_2SO_4:acetone) and three times in dry acetone. Proteins were routinely analysed by electrophoresis in SDS–polyacrylamide gels (Laemmli, 1970). When proteins were analysed on AUT gels (Bonner *et al.*, 1980) the pellet was initially resuspended in 500 μl dH_20 and centrifuged using microconcentrators (Amicon) for 30 min at 13 000 r.p.m. This step was repeated (to remove residual SDS) and 2 volumes of AUT loading buffer (8 M urea, 5% 2-mercaptoethanol, 1 M glacial acetic acid plus a few drops of tracking dye pyroninY) added to the final concentrated sample. AUT gels were silver stained (Irie, 1982; modified by Dorbic and Wittig, 1986). Western blotting, immunostaining and detection by enhanced chemiluminescence (Amersham) or ^{125}I conjugated antibody were carried out as previously described (Turner *et al.*, 1992).

Slot-blot analysis and hybridization

DNA samples were diluted in 0.6 M NaCl to equalize [^{3}H]thymdine counts. All samples were heat denatured at 95°C for 10 min and cooled on ice for 5 min before carrying out five serial doubling dilutions in ice-cold 2 M ammonium acetate. [^{3}H]Thymidine counts were re-tested after heat denaturation. Aliquots (200 μl) of each sample dilution was loaded in duplicate onto Hybond N+ filters (Amersham) using a slot-blot manifold (BioRad). All slots were washed twice with 200 μl 1 M ammonium acetate before fixing the DNA on the filter with 0.4 M NaOH.

Hybridizations were performed as follows, with temperature and hybridization conditions adjusted depending on the DNA probe used. DNA probes (100 ng) were labelled by random priming (Maniatis *et al.*, 1982) using a random oligolabelling kit (Pharmacia) following the manufacturer's instructions. Filters were pre-hybridized at 65°C for at least 4 h in 20 ml 5× SSC, 5× Denhardt's solution, 0.5% SDS plus 0.5 ml 1 mg/ml heat-denatured salmon sperm DNA. Heat-denatured DNA probes were added directly to the pre-hybridization buffer at 1×10^7 c.p.m./ml and hybridized overnight at 65°C. Filters were rinsed in 2× SSC, 0.1% SDS before washing sequentially in 1× SSC, 0.1% SDS for 30 min and 0.5× SSC, 0.1% SDS for 15 min at 65°C. For hybridizations using end-labelled DNA probes (Maniatis *et al.*, 1982) filters were pre-hybridized for at least 4 h at 30–37°C in 6× SSC, 10 mM $NaHPO_4$, pH 6.8, 1 mM EDTA, pH 8, 0.5% SDS, 0.1% non-fat dried milk before addition of DNA probe at 10^6–10^7 c.p.m./ml. After overnight hybridization the filters were rinsed in 2× SSC and then washed sequentially for 10 min at 37°C in: 2× SSC, 0.01% SDS; 1× SSC, 0.01% SDS; 0.3× SSC, 0.01% SDS. Filters, wrapped in Saran wrap, were autoradiographed with Hyperfilm+ intensifying screen at –80°C. Filters previously labelled with DNA probes were stripped by pouring boiling 0.5% SDS over the filters and cooling gradually to room temperature. Completeness of stripping was checked by overnight autoradiography. Relative intensities of the slots obtained after hybridization were initially determined by laser densitometry using an LKB Ultroscan XL laser densitometer and later using a PhosphorImager (Molecular Dynamics).

DNA probes

c-*myc*, clone pDIRH19, was a gift from Dr Terry Rabbitts (Laboratory of Molecular Biology, MRC, Cambridge, UK). pDIRH19 is an unpublished clone of the human c-*myc* *Hin*dIII–*Eco*RI genomic fragment derived from λD1 (Rabbitts *et al.*, 1984). Various restriction enzyme digestions were performed to generate c-*myc* subclones, as detailed in Figure 4. c-*fos*, clone pc-fos3 (mouse), was a gift from Dr Alan Johnstone (St George's Hospital Medical School, London, UK) and was digested with *Bam*HI and *Eco*RI to generate a 3.4 kb fragment containing three of the four exons (van Beveren *et al.*, 1983). ε-Globin, clone pε1.3 (human), was a gift from Dr John Old (Radcliffe Infirmary, Oxford, UK) and was digested with *Bam*HI and *Eco*RI to generate a 1.3 kb fragment. β-Actin (mouse) was a gift from Dr David Huen (Cancer Studies, Birmingham University, Birmingham, UK) and was digested with *Pst*I to generate 1.1 kb fragment. Proinsulin (human), 500 bp *Eco*RI fragment, was a gift from Dr Kevin Docherty (Aberdeen University, Aberdeen, UK). Heterochromatin probes HeT266 [5′-$(CCATT)_n$-3′, where $n = 6$] and HeT405 (5′-GAA GAA GCT TTC TGA GAA ACT GCT TAG TG-3′) were gifts from Dr Arthur Mitchell (MRC Human Genetics Unit, Edinburgh, UK). Telomeric DNA probe C-36 [5′-$(CCCTAA)_n$-3′, where $n = 6$] was a gift from Dr Titia de Lange (Rockefeller University, New York, NY). Restriction enzymes used to isolate DNA probes from the above clones were all purchased from Gibco BRL and Pharmacia.

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References

Alfageme,C.R., Zweider,A., Mahowald,A. and Cohen,L.H. (1974) *J. Biol. Chem.*, **249**, 3729–3736.

Allegra,P., Sterner,R., Clayton,D.F. and Allfrey,V.G. (1987) *J. Mol. Biol.*, **196**, 379–388.

Allfrey,V.G., Faulkner,R. and Mirskey,A.E. (1964) *Proc. Natl Acad. Sci. USA*, **51**, 786–794.

Boffa,L.C., Walker,J., Chen,T.A., Sterner,R., Mariani,R. and Allfrey,V.G. (1990) *Eur. J. Biochem.*, **194**, 811–823.

Bonner,W.M., West,M.H.P. and Stedman,J.D. (1980) *Eur. J. Biochem.*, **109**, 17–23.

Braunstein,M., Rose,A.B., Holmes,S.G., Allis,C.D. and Broach,J.R. (1993) *Genes Dev.*, **7**, 592–604.

Chen,T.A. and Allfrey,V.G. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 5252–5256.

Chen,T.A., Sterner,R., Cozzolino,A. and Allfrey,V.G. (1990) *J. Biol. Chem.*, **212**, 481–493.

Clayton,A.L., Hebbes,T.R., Thorne,A.W. and Crane-Robinson,C. (1993) *FEBS Lett.*, **336**, 23–26.

Collins,S.J., Ruscetti,F.W., Gallagher,R.E. and Gallo,R.C. (1978) *Proc. Natl Acad. Sci. USA*, **75**, 2458–2462.

Craig,J.M. and Bickmore,W.A. (1994) *Nature Genet.*, **7**, 376–381.

Dorbic,T. and Wittig,B. (1986) *Nucleic Acids Res.*, **14**, 3363–3673.

Durrin,L.K., Mann,R.K., Kayne,P.S. and Grunstein,M. (1991) *Cell*, **65**, 1023–1031.

Hebbes,T.R., Thorne,A.W. and Crane-Robinson,C. (1988) *EMBO J.*, **7**, 1395–13–1403.

Hebbes,T.R., Thorne,A.W., Clayton,A.L. and Crane-Robinson,C. (1992) *Nucleic Acids Res.*, **20**, 1017–1022.

Hebbes,T.R., Clayton,A.L., Thorne,A.W. and Crane-Robinson,C. (1994) *EMBO J.*, **13**, 1823–1830.

Ip,Y.T., Jackson,V., Meiers,J. and Chalkley,R. (1988) *J. Biol. Chem.*, **236**, 14044–14052.

Irie,S., Sezaki,M. and Kato,Y. (1982) *Anal. Biochem.*, **126**, 350–354.

Jeppessen,P. and Turner,B.M. (1993) *Cell*, **74**, 281–289.

Jeppesen,P., Mitchell,A., Turner,B.M. and Perry,P. (1991) *Chromosoma*, **100**, 322–332.
Johnson,L.M., Fisher-Adams,G. and Grunstein,M. (1992) *EMBO J.*, **11**, 2201–2209.
Kamakaka,R.T. and Thomas,J.O. (1990) *EMBO J.*, **9**, 3997–4006.
Kayne,P.S., Kim,U.-J., Han,M., Mullen,J.R., Yoshizaki,F. and Grunstein,M. (1988) *Cell*, **55**, 27–39.
Krumm,A., Meulia,T., Brunvard,M. and Groudine,M. (1992) *Genes Dev.*, **96**, 2201–2213.
Laemmli,U.K. (1970) *Nature*, **277**, 680–683.
Loidl,P. (1994) *Chromosoma*, **103**, 441–449.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mann,R.K. and Grunstein,M. (1992) *EMBO J.*, **11**, 3297–3306.
Mathew,C.G.P., Goodwin,G.H. and Johns,E.W. (1979) *Nucleic Acids Res.*, **6**, 167–179.
Mathis,D., Ouet,P. and Chambon,P. (1980) *Prog. Nucleic Acid Res. Mol. Biol.*, **24**, 1–55.
Mendelson,E., Landsman,D., Druckmann,S. and Bustin,M. (1986) *Eur. J. Biochem.*, **160**, 253–260.
Muller,R., Curran,T., Muller,D. and Guilbert,L. (1984) *Nature (Lond.)*, **314**, 546–548.
Perry,M. and Chalkley,R. (1981) *J. Biol. Chem.*, **256**, 3313–3318.
Rabbitts,T.H., Forster,A., Hamlyn,P. and Baer,R. (1984) *Nature*, **309**, 592–594.
Schatz,D.G., Oettinger,M.A. and Baltimore,D. (1989) *Cell*, **53**, 1035–1048.
Schlake,T., Klehr-Wirth,D., Yoshida,M., Beppu,T. and Bode,J. (1994) *Biochemistry*, **33**, 4197–4206.
Siebenlist,U., Bressler,P. and Kelly,K. (1988) *Mol. Cell. Biol.*, **8**, 867–874.
Strobl,L.J. and Eick,D. (1991) *EMBO J.*, **11**, 3307–3314.
Tazi,J. and Bird,A (1990) *Cell*, **60**, 909–920.
Thorne,A.W., Kmiciek,D., Mitchelson,K., Sautiere,P. and Crane-Robinson,C. (1990) *Eur. J. Biochem.*, **191**, 701–713.
Turner,B.M. (1991) *J. Cell Sci.*, **99**, 13–20.
Turner,B.M. (1993) *Cell*, **75**, 5–8.
Turner,B.M. and Fellows,G. (1989) *Eur. J. Biochem.*, **179**, 131–139.
Turner,B.M. and O'Neill,L.P. (1995) *Semin. Cell Biol.*, in press.
Turner,B.M., O'Neill,L.P. and Allan,I.M. (1989) *FEBS Lett.*, **253**, 141–145.
Turner,B.M., Birley,A. and Lavender,J. (1992) *Cell*, **69**, 375–384.
van Beveren,C., van Straaten,F., Curran,T., Muller,R. and Verma,I.M. (1983) *Cell*, **32**, 1241–1255.
Weintraub,H. and Groudine,M. (1976) *Science*, **193**, 848–856.

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